0022-202X/85/8403-0180$02.00/0
THE JOURNAL OF INVESTIGATIVE DERMATOLOGY, 84:180–183, 1985

Vol. 84, No. 3
Printed in U.S.A.

Percutaneous Absorption of Nitroaromatic Compounds: In Vivo and In Vitro Studies in the Human and Monkey

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The percutaneous absorption of 2-nitro-*p*-phenylenediamine, 4-amino-2-nitrophenol, nitrobenzene, *p*-nitroaniline, and 2,4-dinitrochlorobenzene was measured through human and monkey skin. Human studies were performed with excised skin in diffusion cells. Absorption through monkey skin was measured by in vivo and in vitro techniques. Results were compared with those from previously reported human in vivo studies on 2,4-dinitrochlorobenzene and nitrobenzene. Rapid penetration was observed with all compounds, with maximum absorption occurring in the first few hours. No significant differences in absorption were found in values obtained by the different procedures except for the highly volatile (and therefore difficult to compare) compound nitrobenzene. A comparison of the human and monkey in vitro data showed a trend toward increased absorption through monkey skin, but the increase was not statistically significant. The monkey in vivo and in vitro results showed that absorption of all compounds except nitrobenzene was slightly less in the in vitro studies; however, the values were not significantly different. The relative volatility of these nitroaromatic compounds was measured by the loss of compound from epidermal discs at various time intervals. The greatest loss of applied material occurred with nitrobenzene; however, substantial amounts of the other compounds were lost, particularly during the first minute after application as the acetone vehicle evaporated. Monkey skin was found to be a good model for human skin for the determination of the percutaneous absorption of these compounds, and in vitro measurements of absorption agreed reasonably well with values obtained by in vivo techniques. A good correlation was not observed between the absorption of these compounds and their solubility properties.

A number of nitroaromatic compounds are of concern from a toxicologic standpoint because of their potential absorption through the skin of exposed individuals. Several nitroaromatic hair dyes that are carcinogenic in animal feeding studies are known to be absorbed through human skin [1]. The skin is a principal route of exposure of industrial workers to nitroaromatic compounds; even with exposure to only the vapor of nitrobenzene, about one-third of the absorbed dose in a clothed man was the result of skin penetration [2]. Because of dermal absorption, air sample analysis alone is insufficient to protect workers from the cyanogenic effect of nitroaromatic compounds [3].

The absorption of only a limited number of nitroaromatic compounds has been quantitated. Nitrobenzene penetration through human skin was measured from both the liquid [4] and vapor [5] states. Feldmann and Maibach [6] determined the percentages of the applied dose absorbed in living humans for 2,4-dinitrochlorobenzene and nitrobenzene. 2,4-Dinitrochlorobenzene penetrated both protective gloves and skin to form methemoglobin in a human volunteer [7]. The absorption of several nitroaromatic hair dyes through human and animal skin has been measured [1,8,9].

Five common nitroaromatic compounds were chosen for study under similar conditions in human and monkey skin. In addition to obtaining information about the permeability of these compounds, other primary goals were to compare the permeability properties of human and monkey skin and to determine whether results obtained by in vivo and in vitro procedures were similar.

Dosing was performed using "finite" amounts of the compounds in an acetone vehicle for calculation of the percentage of the applied amount absorbed. This method was considered more relevant to exposure conditions than application of "infinite" amounts in solution for calculation of permeability constants. It is also the method of choice for comparing in vivo and in vitro absorption data, as permeability constants are difficult to obtain by in vivo techniques.

Absorption through human skin was measured by using excised skin in diffusion cells, and absorption through monkey skin was determined by both in vivo and in vitro techniques. The relative volatility of each compound was quantitated in separate experiments by measuring the loss of radioactivity from waxed paper or epidermal discs, and results were considered in the interpretation of percutaneous absorption data.

MATERIALS AND METHODS

^{14}C-Labeled radioisotopes were utilized for both absorption and volatility measurements. New England Nuclear (Boston, Massachusetts) supplied 2-nitro-*p*-phenylenediamine (sp act, 1.32 mCi/mmol). ICN Inc. (Irvine, California) synthesized 4-amino-2-nitrophenol (10 mCi/mmol). Nitrobenzene (6.6 mCi/mmol), *p*-nitroaniline (15.4 mCi/mmol), and 2,4-dinitrochlorobenzene (13.3 mCi/mmol) were supplied by Pathfinder Laboratories (St. Louis, Missouri). The radiochemical purity of each compound was equal to or greater than 97%.

In vivo monkey skin absorption measurements were performed according to the procedure of Feldmann and Maibach [10]. Each compound was applied to a shaved area of abdominal skin in an acetone vehicle at a concentration of 4 $\mu g/cm^2$. Monkeys were restrained in metabolic chairs so that the amount of compound excreted in the urine in a 5-day period could be determined. Results were corrected for excretion by routes other than the urine by determining, in separate experiments, the percentage of a parenteral dose that was recovered in the urine in the same time period.

The absorption through excised human and monkey abdominal skin was measured by in vitro diffusion cell techniques. In some experiments, static diffusion cell methodology was used with aliquots removed periodically from the receptor of cells with a skin surface area of 1.13 cm^2 [11]. In other experiments, a newly designed flow-through cell [12] was used to obtain continuous monitoring for absorption profiles (Fig 1); normal saline was pumped through the cells (skin surface area, 0.64 cm^2) at a rate of approximately 5 ml/h and collected in scintillation vials for counting. Similar absorption values are obtained with either diffusion cell procedure [12]. Monkey skin was lightly shaved with electric clippers as in the in vivo experiments, using care to prevent damage to the skin [13]. Dermatome sections of human and monkey skin were utilized in the permeability studies. The upper 350 μm of skin was removed from the skin surface with a Padgett Electro Der-

Manuscript received March 9, 1984; accepted for publication August 9, 1984.

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matome. As in the in vivo monkey experiments, compounds were applied to skin in an acetone vehicle at a concentration of 4 $\mu g/cm^2$. Some diffusion cells with nitrobenzene were occluded with Parafilm to measure the increase in absorption when evaporation was prevented. The site of application was washed with soap and water at 24 h in all experiments. Absorbed radioactivity was determined by liquid scintillation counting with a Beckman LS-9000 instrument.

The volatility of the nitroaromatic compounds was determined by measuring their evaporation from 2 surfaces, waxed paper and human epidermis. Epidermal sheets were prepared by submerging full-thickness skin in 60°C water for 0.5 min and then peeling off the upper layer. Approximately 1 μg of each compound in 3 μl of acetone was applied to circular discs (1-cm diameter) punched from the waxed paper or epidermis. Each disc was then placed inside a diffusion cell top so that the air currents across its surface would be similar to those in the in vitro permeability experiments. At various times after application of the compound, discs were added to scintillation fluid and the amount of remaining radioactivity was determined.

Octanol/water partition coefficients were determined by adding approximately 1 μCi of compound to a mixture containing 5 ml each of octanol and water. The container was sealed tightly and shaken for 24 h. After the phases had separated, aliquots were removed from each to determine partitioning.

Results were statistically analyzed by Student's t-test. The significance of the difference between absorption values was determined by a 2-tailed probability test, $p < 0.05$.

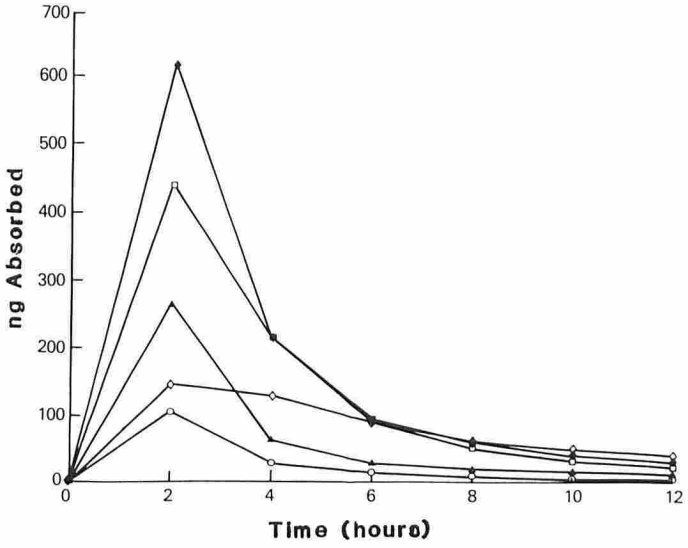


FIG 1. Absorption profiles of the nitroaromatic compounds. Absorption values from flow-through cells only are plotted to show the rapid occurrence of the maximum absorption of each compound. Each point represents the mean of the amount of compound determined at each 2-h sample collection time. □ = p-Nitroaniline; ◆ = 4-amino-2-nitrophenol; ▲ = 2,4-dinitrochlorobenzene; ◇ = 2 nitro-p-phenylenediamine; ○ = nitrobenzene.

RESULTS

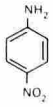
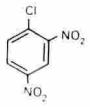
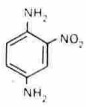
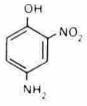
Physical chemical properties of this homologous series of nitroaromatic compounds are given in Table I. In general, the molecular weights of these compounds are similar. The water and octanol solubility properties vary substantially, with approximately a 25-fold range existing in the respective octanol/water partition coefficients.

The percutaneous absorption measurements were made by following a standard protocol so that the vitro experiments performed in the Food and Drug Administration laboratories could be compared with the in vivo experiments performed at the University of California, both in the current and previous studies [6].

The urinary excretion of a parenteral dose of each compound was determined in monkeys as described in *Materials and Methods* to correct for excretion by routes other than the urine. These values were: p-nitroaniline, 86.7%; 4-amino-2-nitrophenol, 68.3%; 2,4-dinitrochlorobenzene, 85.8%; and nitrobenzene, 81.4%.

The combined absorption data for the human and monkey studies by in vivo and in vitro techniques are given in Table II.

TABLE I. *Chemical properties of nitroaromatic compounds*

Compound	Structure	Molecular weight[a]	Water solubility (g/liter)	Octanol solubility (g/liter)[b]	Partition coefficient, octanol/water[c]	Melting point (°C)[a]
Nitrobenzene		123	2.0[a]	141.6	70.8	6
p-Nitroaniline		138	0.8[a]	19.6	24.5	148
2,4 Dinitrochlorobenzene		203	0.08[d]	6.4	80.0	53
2-Nitro-p-phenylenediamine		153	1.8[d]	6.1	3.4	137
4-Amino-2-nitrophenol		153	0.6[d]	5.5	9.1	131

[a] *The Merck Index*, Merck and Co., 1976.
[b] Values calculated from the water solubility and octanol/water partition coefficients.
[c] Determined as described in *Materials and Methods*.
[d] Determined from solubility in water of a saturated solution.

TABLE II. *Percutaneous absorption of nitroaromatic compounds*

Compound	Percent applied dose			
	Human		Monkey	
	In vivo[a]	In vitro	In vivo	In vitro
p-Nitroaniline		48.0 ± 11.0 (9)	76.2 ± 8.4 (4)	62.2 ± 6.1 (6)
4-Amino-2-nitrophenol		45.1 ± 8.0 (5)	64.0 ± 6.2 (6)	48.2 ± 7.8 (5)
2,4-Dinitrochlorobenzene	53.1 ± 6.2 (4)	32.5 ± 8.7 (8)	52.5 ± 4.3 (4)	48.4 ± 3.9 (11)
2-Nitro-*p*-phenylenediamine		21.7 ± 2.6 (7)	29.9 ± 6.9 (3)[b]	29.6 ± 4.3 (5)
Nitrobenzene	1.5 ± 0.3 (6)	7.8 ± 1.2 (6)	4.2 ± 0.5 (4)	6.2 ± 1.0 (6)
		41.1 ± 2.0 (3)[c]		

Values are the means ± SE of the number of determinations in parentheses. Only with nitrobenzene were there significant differences (Student's 2-tailed *t*-test, $p < 0.05$) among the values determined for the compounds by the 4 different methods; the value for the human in vivo study was significantly different than the results from the other 3 procedures, and there also was a significant difference between the human in vitro and the monkey in vivo values.

[a] Values from [6].

[b] Value from [9].

[c] Diffusion cell tops were covered with Parafilm.

TABLE III. *Evaporation of nitroaromatic compounds*

Compound	Percent applied dose remaining at:					
	1 min[a]		3 h		24 h	
	P	E	P	E	P	E
p-Nitroaniline	53.4	84.3	49.9	72.8	55.0	77.5
4-Amino-2-nitrophenol	54.9	80.6	53.6	85.4	54.1	70.2
2,4-Dinitrochlorobenzene	98.8	65.0	33.0	68.3	19.1	57.0
2-Nitro-*p*-phenylenediamine	75.7	93.3	79.7	90.6	85.6	86.1
Nitrobenzene	19.8	75.8	8.6	30.4	7.8	21.9

Acetone solutions (3 μl) were applied to waxed paper (P) or human epidermal (E) discs. The amount remaining on the discs was measured at the indicated times. The results are the average of 3 determinations.

[a] Acetone evaporates.

The compounds are listed in the general order of decreasing permeability. In only one case was there an exception to this similarity in the order of ranking and that occurred in the in vitro monkey data; 2,4-dinitrochlorobenzene absorption was barely (although not significantly) greater than the absorption of 4-amino-2-nitrophenol.

The time course of absorption of these compounds can best be seen in the absorption profiles from in vitro data obtained with human skin in the flow-through diffusion cells (Fig 1). The maximum absorption rate for all compounds occurred within the first 2 h after application to the skin. The absorption decreased rapidly in subsequent samples and was essentially complete by the end of 24 h.

Significant amounts of the applied compounds were unaccounted for and did not penetrate the skin. However, when diffusion cells were occluded with Parafilm to prevent nitrobenzene evaporation, total absorption was increased more than 5-fold (Table II). The relative volatility of these substances was therefore measured as an aid in interpreting the absorption data. The amount of radioactivity remaining on circles punched from waxed paper or human epidermis was determined at various times after application (Table III). The percentage of the applied dose remaining on the epidermis was generally greater than that on the paper disc, particularly at the longer time intervals. Evaporation of all the compounds occurred during the first minute as the acetone also evaporated. Substantial loss of nitrobenzene from the epidermal discs, however, occurred not only along with the acetone, but also during the remainder of the 24-h experiment.

DISCUSSION

In vivo absorption of the nitroaromatic compounds was determined in monkeys by the widely used parenteral dose correction technique of Feldmann and Maibach [10]. In spite of the indirect estimate of percutaneous absorption obtained, no more relevant technique has been devised for estimating absorption in humans and expensive animals that cannot be sacrificed at the end of a study. For compounds excreted primarily in the feces (not the compounds in this study), a summation of the chemical found in the feces and urine may provide a more accurate estimate of absorption [14]. Shah and Guthrie [15] have recently obtained a good correlation in pesticide absorption measurements by using the parenteral correction technique and a direct method which consisted of the summation of compound found in the skin of the application site, urine, feces, blood, liver, and carcass.

The nitroaromatic compounds were rapidly, although not completely, absorbed through human and monkey skin. The absorption of the 5 compounds through human skin was measured only with excised skin in diffusion cells; the permeation of 2,4-dinitrochlorobenzene and nitrobenzene in vivo through human skin had previously been determined [6]. Values for the in vivo 2,4-dinitrochlorobenzene absorption more closely resembled results from the monkey skin experiments, but there was no significant difference among any of the values for this substance.

Values for nitrobenzene, the other compound evaluated by all procedures, were lower in the human volunteer study than with either human or monkey skin in the current study; however, comparison is difficult for several reasons. Nitrobenzene is highly volatile and the environment immediately around the application site can affect the amount absorbed. For this reason in particular, valid conclusions regarding in vivo and in vitro comparisons are difficult to make with this compound. In addition, there is evidence that the abdominal skin of humans is more permeable than forearm skin for several pesticides (malathion, 1.4 times; parathion, 2.1 times) [16]. Regional variability in skin permeation also occurs in the monkey [17], and we have found that 4-amino-2-nitrophenol is absorbed 1.3 times more readily through abdominal skin than through forearm skin in the monkey (data not shown). In the human volunteer studies (Table II), forearm skin was used but abdominal skin was used throughout the present study, so the lower absorption might be at least partially the result of this difference. A comparison of the in vitro data from humans and monkeys shows that penetration was greater through monkey skin for all compounds except the highly volatile nitrobenzene. The differences between the two types of skin, however, were not significant (except for nitrobenzene) and results support the opinion that monkey skin is a reasonable animal model for human skin. Comparisons of the permeability of human and monkey skin with other compounds have resulted in similar conclusions [13]. Unfortunately, the comparative data base still remains limited and additional studies need to be performed.

In vivo and in vitro results can be more thoroughly compared with the monkey skin data. Although the in vitro values were

slightly lower (except for nitrobenzene), there was no significant difference among the values determined for the 5 compounds. It should be noted that all the compounds tested are rapidly absorbed and that factors of difference between in vivo and in vitro values in humans and monkeys cannot be large when high percentage absorption occurs. These comparisons with additional compounds provide further evidence to support the conclusion reached in previous comparisons of in vivo and in vitro data [18,19]. Thus, reasonably accurate values for skin absorption of many compounds probably can be obtained by in vitro techniques. The permeation of certain types of chemicals can be difficult to measure accurately by in vitro procedures; for example, hydrophobic compounds may not partition freely into an aqueous diffusion cell receptor fluid. A nonionic surfactant solution has been used in the receptor to facilitate the measurement of absorption of this type of chemical [20].

The maximum rate of absorption of all the compounds, as measured in vitro with human skin, occurred within the first several hours (Fig 1), and for nitrobenzene an initial rate of 82 ng/cm^2/h was calculated (105 ng at sample time/2h/0.64 cm^2 skin area). Salmowa and Piotrowski [4] applied 15 mg of nitrobenzene/cm^2 on skin of human volunteers and measured an absorption velocity of 0.25–3.19 mg/cm^2/h during a similar time period. Therefore, the application of approximately a 4000-fold higher dose in their experiments resulted in a corresponding increase in absorption rate.

The volatility of at least some of these compounds was expected to have an effect on the amount of the applied dose that was eventually absorbed. Nitrobenzene was the only liquid used (see melting points, Table I) and is known to be a volatile compound. Occlusion of some of the diffusion cells in the human skin studies greatly increased absorption of this compound. The amount of the nitroaromatic substances lost after application in acetone to two different surfaces was determined (Table III). Initially, evaporation was measured from circular waxed paper discs that served as a readily available inert surface. The loss due to evaporation was subsequently determined after application to circular discs of human epidermis, which is a more physiologic surface allowing interactions to occur with the applied compound. In general, evaporation was less from the epidermal surface and these values were used for comparative purposes (Table III). Significant evaporation was observed with all 5 of the compounds at each of the measurement times; for the 4 solid substances, most of the loss due to volatility appeared to occur during the first minute as the acetone was evaporating. The loss of nitrobenzene, however, was also substantial at later intervals and only 22% of the applied dose remained on the epidermal disc at the end of 24 h.

The loss of these compounds from the surface of skin is an important consideration for several reasons. Nitrobenzene appeared to be a poorly penetrating compound based on the percentage of the applied dose that is absorbed through the skin (Table II). In fact, the nitrobenzene molecule would be expected to penetrate readily because of its solubility properties (Table I), and its poor penetration is likely due to substantial loss from the surface by evaporation. These results with the solid nitroaromatic compounds indicate that the possible loss of a portion of the compound as the vehicle evaporates should be a concern when substances in volatile vehicles are applied to skin. The frequency of the occurrence of this effect with other chemicals is not known.

In general terms, a correlation of lipid/water partition coefficients and skin permeability is known to exist [21]. When the octanol/water partition coefficients in Table I are compared with the percutaneous absorption values of Table II, a good correlation with these compounds does not appear to occur. This lack of correlation is partly the result of the volatility of these substances, as nitrobenzene should be ranked higher on its ability to penetrate skin. However, the comparison also indicates the limited usefulness of solubility data when used alone in predicting skin absorption.

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